PATENT
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Applicant:	Gerald Krystal et al.	Art Unit:	1636
Serial No.:	09/919,703	Examiner:	R. Schwartzman
Filed:	July 31, 2001	Customer No.:	21559
Title:	NOVEL PEPTIDES AND THEIR USE TO AMELIORATE CELL DEATH		

Assistant Commissioner for Patents
Washington, D.C. 20231

DECLARATION UNDER 37 C.F.R. ə 1.132 OF SIMON W. RABKIN, M.D.

1. I, Simon W. Rabkin, am an inventor on the above-captioned patent application. My qualifications include more than 20 years as a research scientist in the field of cardiovascular disease.

2. I have read the Final Office Action mailed January 2, 2001, filed in the parent case, U.S.S.N. 09/294,457, and submit the following declaration in support of the instant application.

3. As is described in detail in paragraphs 4-10, below, peptides of the instant invention (i.e., those from a streptokinase or comprising the Val-Asp-Val (VDV) sequence) provide a protective effect against cell death when tested *in vitro* on cardiomyocytes of chick, neonatal mouse, and adult rat, and when tested *in vivo* in a rat coronary artery ligation model. Based on the broad spectrum of warm-blooded animals

afforded protection by these peptides, it is highly likely that the peptides will also prevent the death of cardiomyocytes of other warm-blooded mammals, including humans.

4. Treatment of *in vitro* cultured adult rat cardiomyocytes with one of the following peptides: SVDVEY (SEQ ID NO: 1), YVDVDT (SEQ ID NO: 2), and TVDVEY (SEQ ID NO: 3), at a concentration of 10^{-4}M, following exposure to hydrogen peroxide (an agent known to induce apoptosis), reduced the extent of cell death by between 38 and 42%, as determined by an MTT assay (Exhibit A; Table 1).

5. Treatment of *in vitro* cultured adult rat cardiomyocytes with one of the following peptides: SVDVEY (SEQ ID NO: 1), YVDVDT (SEQ ID NO: 2), and TVDVEY (SEQ ID NO: 3), at a concentration of 10^{-4}M, following exposure to sodium azide (another agent known to induce apoptosis), reduced the extent of cell death by between 77 and 100%, as determined by the MTT assay (Exhibit A; Table 2).

6. Cultured chick ventricular cardiomyocytes were treated with ammonium persulfate (APS; 100 mM), yet another agent known to induce cell death, and the extent of cell death ascertained by the trypan blue assay. The protective effect of SVDVEY was demonstrated as it reduced the percentage of dead cells per high power field from 70% to 42% (Exhibit B). The beneficial effect of SVDVEY is related in part to the peptide VDV sequence, as amino acid substitutions in this region reduced the effectiveness of the protection against APS-induced cell death (Exhibit B).

7. *In vitro* cultured chick ventricular cardiomyocytes were treated with the peptide SVDVEY (SEQ ID NO: 1) and exposed to camptothecin (100 μM) or APS(10 mM). Camptothecin is well known to induce apoptosis. The peptides, tested at concentrations of 10^{-4}M, 10^{-6}M, and 10^{-8}M, provided protection against apoptosis. Apoptosis was determined by a Roche assay, which detects the typical pattern of DNA fragmentation during apoptosis by measuring the absorbance of DNA at OD470. The data indicate that the amount of DNA fragmentation decreased with increasing peptide concentration (Exhibit C; Table 1 and 2).

8. *In vitro* cultured neonatal mouse ventricular cardiomyocytes were treated with the peptide TVDVEY (SEQ ID NO: 3) and exposed to APS (100 mM). Apoptosis was determined by the Roche assay. The data indicate that the amount of DNA fragmentation was decreased by the peptide, even at a high APS concentration that induced over a two - fold increase in the amount of apoptosis (Exhibit C; Table 3). The peptide reduced apoptosis by 27.6%.

9. Besides providing a clear protective effect *in vitro*, the peptides of the instant invention provide a protective effect against cell death *in vivo* as well. The specification teaches that the treatment of an isolated intact rat heart with a 20-mer peptide containing the SVDVEY sequence (SEQ ID NO: 6) permitted more rapid recovery, as measured by ventricular pressure, after prolonged ischemia (see page 25, line 14-20, and page 26 of the specification).

10. In an example of *in vivo* inhibition of myocardial infarction-mediated cell death induced by coronary artery ligation in the intact rat, treatment with any one of the peptides SVDVEY (SEQ ID NO: 1), YVDVDT (SEQ ID NO: 2), and TVDVEY (SEQ ID NO: 3)

protected the heart from damage, as determined by quantification of TUNEL-positive nuclei (Exhibit D) or by examination of the amount of infarcted (dead) heart as assessed by the Evan's blue/TTC method (Exhibit E). Although the volume of heart deprived of blood was the same in the control heart and the treated heart, the amount of infarct (pale area) was reduced by between 48 and 62% in the heart treated with one of the peptides, as compared to the control heart administered diluent alone. These data indicate that the peptides prevented ischemia/reperfusion-induced cell death associated with coronary occlusion.

11. The rat coronary ligation model is an excellent and well-accepted model for the study of human cardiovascular diseases and therapies. This is true because the rat cardiovascular and cellular processes that affect cardiovascular degeneration are very closely related to the cardiovascular and cellular processes that govern the process of cardiovascular degeneration in humans. Several investigators have reported the use of coronary artery ligation in the rat to demonstrate the efficacy of a drug in the treatment of human cardiovascular disease. In one example, the rat coronary artery ligation model was used to test angiotensin converting enzyme inhibitors, now administered to many patients after myocardial infarction (see for example, Pfeffer et al., Circulation 72:406-412, 1985; Exhibit F; Pfeffer et al., Circulation 75:I149-155, 1987; Exhibit G). The utility of beta blockers, now administered to many patients after myocardial infarction, was also established in experimental models of coronary artery ligation (see Rasmussen et al., Circulation 56(5):794-798, 1977; Exhibit H).

12. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like

so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Date: April 5/02

Simon W. Rabkin, M.D.